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Scientific Officer
A.J. Melaragno, CAPT, MC, USN
Naval Medical R&D Command
Director of Research and Development
Bethesda, MD 20814-50440

Ref: N00014-91-C-0044

Dear Captain Melaragno:

Enclosed is the Second Triannual Report for Contract No.: N00014-91-C-0044, which is entitled "Cellular and Tissue Injury During Nonfreezing Cold Injury and Frostbite". This Report covers the period from May - August, 1991. If you have any questions about the Report or the research, please contact me at 404-952-1660.

Sincerely,

John F. Carpenter, Ph.D.

cc: Mrs. Mellars, DCMDS-GAACA
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Contract No. N000014-91-C-0044 **Start Date: 31 JAN 1991**

Cellular and Tissue Injury During Nonfreezing Cold Injury and Frostbite

Second Triannual Report: May - August, 1991

Introduction. Below we outline further results from studies testing the effects of stresses encountered during nonfreezing cold injury (e.g., low temperature, acidosis, anoxia, elevated inorganic phosphate levels, and intracellular ionic imbalance) on model systems. As we establish the acute effects, we will begin to investigate the mechanism(s) of irreversible damage induced by long-term exposure to these conditions. In addition, we have started to apply the methodology to sensitive cells and formed blood elements that are involved in cold injury.

Metabolic studies. We chose human red blood cells as the first model for the study of the metabolic consequences of hypothermia and acidosis. Our hypothesis is that low pH and temperatures will synergistically inhibit glycolysis.

We have designed a buffer system that allows us to modulate the intracellular pH (pHi) of red cells across the range from pHi 8.0 to 6.5. We determined the metabolic heat rate with microcalorimetry at 7 different pHi values across this range, for cells incubated at: 37, 30, 25, 20, 15 and 10°C. For example, the heat rate at 37°C and pHi is about 130 microwatts/ml packed red cells. This value decreases to 40 microwatts at pHi 6.5. Metabolic heat rates in the presence of fluoride at 37°C and pHi of 8.0 gave values essentially identical to those noted at pHi 6.5 without fluoride. In contrast, fluoride has no detectable effect on the metabolic heat rate at a pHi of 6.5. Fluoride is a specific inhibitor of glycolysis. Thus, these results indicate that the pHi-induced inhibition of metabolic heat rate in red cells is due to pH-induced inhibition of glycolysis. In addition, they suggest that at pHi 6.5, glycolysis is completely inhibited.

The residual heat noted at pHi 6.5 and in the presence of fluoride has been attributed to the pentose phosphate shunt. Our results suggest that this component of red cell metabolism is insensitive to changes in intracellular pH, across the range that we have tested.

Comparison of metabolic heat rates as a function of intracellular pH at each temperature showed that, within the uncertainty of the heat rate measurements, the relative inhibition at a given intracellular pH did not change with temperature. These results appear to refute our hypothesis. However, the microcalorimetry data can only be compared down to 20°C, because red cells do not produce heat at sufficient rates at lower temperatures. It may be that comparison across a broader temperature range is needed for increased sensitivity to pHi at reduced temperature to be manifested. To this end, we have begun crossover point analysis of metabolites to determine the effects of pHi on activity of key enzymes of glycolysis at 37°C vs. 4°C.

Membrane biophysics and function. We now have our Fourier transform infrared spectrometer set up and operating. With the attached IR microscope, we have been able to measure the thermotropic membrane phase changes in intact cells, human platelets and cardiac muscle sarcoplasmic reticulum vesicles. These values are obtained by determining the vibrational frequency of CH_2 groups (the signals from which are dominated by the hydrocarbon tails of membrane phospholipids) as a function of temperature. As temperature is lowered there is an abrupt decrease in vibrational frequency as the membranes undergo a transition from the liquid crystalline phase to the gel phase. Such phase transitions may be responsible, either directly or indirectly, for hypothermic damage to cells and formed blood elements.

Direct damage includes leakage of contents from the cell and organelles as the delimiting membranes are going through the phase change. Indirect damage can be linked to the depression of transmembrane ionic pump activity that is induced in the gel state. The concomitant loss of ionic gradients and membrane voltage potential are thought to lead to increased intracellular levels of calcium ion. Both leakage from organelles and influx from the external milieu via voltage-gated calcium channels may be involved. The consequences of the disruption of calcium homeostasis include activation of proteases and phospholipases, which can lead to irreversible cell damage. Our goal is characterize this series of events and determine how membrane phase changes may serve as the primary lesion leading to this, ultimately lethal, cascade.

The first model system for these studies is human platelets. We will determine the roles of cold, ischemia and acidosis on platelet function and as inducers of damage to these blood elements. In nonfreezing cold injury the loss of perfusion through the microvasculature is most likely related to platelet aggregation and the concomitant thrombosis. Chilling damage to and/or irreversible activation of platelets is also of great interest to cardiac surgeons, who have documented platelet damage during hypothermic bypass. This research is also relevant to blood banking, since currently platelets must be stored at room temperature to avoid chilling damage. The platelet experiments are part of a new collaborative effort with V. Paul Addonizio, M.D. and Robert Korn, M.D., Ph.D. from the Department of Surgery at the Temple University Medical Center.

Our first experiments have shown that platelets have an extremely high thermotropic membrane phase transition temperature. The midpoint of the transition from the liquid crystalline to the gel state is at about 25°C. In addition, the membrane is fully in the gel state at 15°C. For comparison, we have determined that a human lymphoma cell line (KG-1A) has a transition temperature around 12°C, and the membrane phospholipids are not fully in the gel state until temperatures around 4°C are reached. The extreme sensitivity of the platelet membranes to hypothermia suggest that membrane phase transitions may play an important role in chilling damage to platelets.

Skeletal muscle fiber function. Our most recent research in this area has concentrated on three different areas, the results from which are outlined below.

1) We have found that the effect of decreased pH on maximal force of skinned mammalian skeletal muscle is very similar in fast-twitch and slow-twitch fibers. Decreasing pH from 7.0 to 6.2 decreases force by about 30% in all muscles tested:

Fast-twitch: rabbit psoas, cat lateral gastrocnemius, monkey gastrocnemius

Slow-twitch: rabbit soleus, cat soleus, cat vastus intermedius, monkey soleus

Based on these studies we conclude that rabbit muscle is an appropriate model of mammalian muscle for experiments, and since rabbit gives the same as monkey, the data are likely to be applicable to humans as well.

2) The synergistic interaction of inorganic phosphate and decreased pH is observed in all mammalian fast-twitch skeletal muscles but not in slow twitch skeletal muscles or heart. These results will be reported (Abstract enclosed) at the 1992 joint meeting of the American Society of Biochemistry and Molecular Biology and the Biophysical Society (9-13 Feb 1992). This is important for muscle function with strenuous exercise and/or hypoxia (such as might occur with impaired blood flow to limb muscle during nonfreezing cold injury) where increased intracellular phosphate and decreased pH will be observed. It is interesting physiologically that slow-twitch postural muscles do not show this synergism, thus they will be able to function better than fast-twitch locomotory muscles under conditions of impaired blood flow. Our experiments to date have been mainly at room temperature (22°C). However, we have found that the synergism between phosphate and decreased pH is rabbit psoas fibers is also observed in the cold (7°C).

3) Increasing ionic strength decreases maximal calcium-activated force of skinned skeletal and cardiac fibers. High ionic strength conditions could arise during and after tissue freezing because solutes are concentrated due to ice formation. The effect on muscle fibers is thought to be due to two mechanisms: electrostatic screening of myosin-actin interaction and destabilization of cross-bridge (i.e., myosin) structure/function. This conclusion is bolstered by the observation that trimethylamine N-oxide (TMAO) can to some extent counteract the depressant effects of high ionic strength, especially in the presence of structure-disrupting salts such as potassium chloride. We have shown this previously in rabbit skeletal muscle (Biophysical J. 57:546a, 1990). We have recently confirmed this for cardiac muscle. This will be reported at the 1992 joint meeting of the American Society for Biochemistry and Molecular Biology and the Biophysical Society (Abstract enclosed). These findings provide an important foundation for our future work which will examine the influence of protein stabilization by TMAO on the response of muscle to decreased pH and temperature.

Potential application of antifreeze peptide to cold injuries. We have proposed that antifreeze peptides could potentially have usefulness in preventing frostbite since they lower the freezing temperature of water. However, the levels needed to achieve this effect are prohibitively high (e.g., > 10 mg/ml); both from an economic and, most likely, medical viewpoint. A few reports have shown that additives such as gelatin can greatly enhance the antifreeze activity of glycoproteins obtained from cold-tolerant insects. We have been working with a recombinant antifreeze peptide from winter flounder. To test whether the enhancement noted with the insect proteins could be seen with our recombinant peptide, we have developed an assay for measuring freezing point depression with differential scanning calorimetry.

The assay revealed that 5, 10, and 20 mg/ml of the recombinant winter flounder

peptide had the ability to prevent ice growth over a temperature range 1.6 to 1.9°C below the solution freezing point. Low concentrations of additives were tested and found to have no ability to prevent ice growth in the absence of the protein. Additives tested include: agar, gelatin, hydroxyethyl starch, dimethyl sulfoxide, and polyethylene glycol. The additives were then mixed with 5 mg/ml antifreeze protein to determine if the antifreeze activity could be potentiated. 0.5% agar, 1% hydroxyethyl starch, and 1% dimethyl sulfoxide did not have any discernable potentiation effect. 0.5% gelatin and 1% polyethylene glycol, however, did reveal an increase in antifreeze protein activity of at least 2°C beyond control levels.